Plant Cell Reports (1986) 5:127–131

Plant Cell Reports

Isolation, culture, and cell division in cotyledon protoplasts of cotton (*Gossypium hirsutum* and *G. barbadense*)

Ebrahim Firoozabady and David L. DeBoer

Agrigenetics Corporation, Advanced Research Division, 5649 East Buckeye Road, Madison, WI 53716, USA

Received September 24, 1985 – Communicated by F. Skoog

ABSTRACT

Protoplasts were isolated from cotyledons and foliage leaves of cotton (Gossypium hirsutum and G. barbadense). Cotyledon protoplasts were larger and responded to culture better than leaf protoplasts. Cotyledon derived protoplasts regenerated cell walls and formed microcolonies of 2-3 cells in G. hirsutum and 5-8 cells in G. barbadense. However, the microcolonies did not grow beyond this stage. Protoplast yield and viability, cell wall regeneration and cell division were influenced by several factors, e.g., genotype, age, tissue and growth condition of donor plant, enzyme mixture and concentration, preplasmolysis period, incubation period, and culture medium.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α-naphthaleneacetic acid; BAP, 6-benzylaminopurine; GA_3, gibberellic acid; pCPA, p-chlorophenoxyacetic acid; MES, 2[N-morpholino]ethanesulfonic acid

INTRODUCTION

In the last decade considerable attention has been paid to the development of protoplast technology. Protoplast isolation and culture are influenced by many factors, e.g., growth conditions of donor plants, preplasmolysis treatments, types and concentrations of enzymes, purification procedures, pH, osmoticum of solutions, etc. (Keller et al. 1982). The systematic analysis of these factors is extremely laborious, empirical, and sometimes unsuccessful. In freshly isolated protolasts, one must also contend with vast changes in their membrane potential (Racusen et al., 1977).

While protoplasts from Gossypium cotyledons (Khasanov and Butenko, 1979) and hypocotyl callus (Bhojwani et al., 1977; Finer and Smith, 1982) have been isolated and cultured, the conditions for isolation and culture of a high number of viable cotyledon protoplasts of Gossypium have not been optimized. In this paper, we report investigations on isolation, culture, and cell division of leaf mesophyll and cotyledon protoplasts of cotton.

MATERIALS AND METHODS

Plant Material. Seeds of G. hirsutum cultivars GSA 20, GSA 71, GSA 78, Rex, RC15, Acala SJ-2, G8160, A, R, and A406 and G. barbadense cultivars Giza 7 and SBSI were surface sterilized for 30 s in 70% ethanol, 15 min in 33% commercial bleach, washed twice with sterile distilled water, germinated on hormone-free MS medium (Murashige and Skoog, 1962), and incubated at 30°C in 16 h daylight of 4,000-5,000 lux provided with flourescent lamps.

Protoplast Isolation. Cotyledons from 10- to 12-day-old seedlings and young leaves of approximately 3-week-old plants were selected for protoplast isolation. Leaves or cotyledons were preincubated on an agar plasmolysis medium, MSG (MS salts, 3% w/v glucose, MS vitamins but with 5 times the concentration of thiamine·HCl, 0.8% agar, pH 5.7) for 24 h prior to enzyme treatments. Tissues were then cut into longitudinal strips (1-2 mm), and placed into Erlenmeyer flasks with an enzyme solution (15 ml/g fresh weight of tissue) containing 1% Cellulysin, 0.5% Macerase (both from Calbiochem, San Diego, CA USA), MS salts, UM vitamins (Uchimiya and Murashige, 1974), 400 mg/l myoinositol, 0.5 mg/l 2,4-D, 1.0 mg/l NAA, 0.25 mg/l BAP, 0.25 mg/l kinetin, 7% w/v mannitol, 2% w/v glucose, 10 mM $CaCl_2$, pH 5.7. To infiltrate the tissues with the enzyme mixture, flasks were evacuated for 20 min using a vacuum dessicator (Bell-Art Products, Pequannock, NJ, USA) connected to a water aspirator, and then incubated on a shaker (50 rpm) at 30°C in the dark for ≈17 h.

Protoplast Purification. Protoplasts were separated from undigested tissues by filtration through 8 layers of cheesecloth, and pelleted by centrifugation at 100 g for 5 min. Pellets of cotyledon protoplasts were resuspended in 10-20 ml of a 20% w/v sucrose solution containing MS salts and vitamins, pH 5.7. For leaf protoplasts, 10% Percoll was added to the same sucrose solution to make it more dense since leaf protoplasts have smaller vacuoles and are heavier. Each protoplast suspension was overlayed with 5 ml of a rinse solution containing MS salts and vitamins, 9% w/v mannitol, pH 5.7 and was centrifuged at 100 g for 10 min. Intact protoplasts free of cell debris were recovered from the interface, diluted with the rinse solution, and pelleted by centrifugation at 100 g for 5 min. This rinse procedure was repeated two more times prior to plating. Protoplast number was estimated with a hemocytometer and protoplast viability was tested by staining with fluorescein diacetate (FDA) (Widholm, 1972).

Offprint requests to: E. Firoozabady

Protoplast Culture. Protoplasts (2.5×10^4/ml) were cultured in a modified MS medium (MSm) containing MS salts supplemented with 5 mM NH_4NO_3, 3 mM $CaCl_2$, 50 µM H_3BO_3 and 30 µM $ZnSO_4$, with UM vitamins and 400 mg/l myoinositol, 7% mannitol, 2% glucose, 0.3% galactose, 0.2 mg/l each of BAP and kinetin, 0.5 mg/l 2,4-D, 1 mg/l NAA, 1 mg/l zeatin and 1 mM glutamine, pH 5.7. The protoplasts were distributed in 5 cm Petri dishes (3 ml/plate), and incubated at 30°C in the dark. The regeneration of cell walls was tested by staining with Calcofluor White as described by Galbraith (1981).

RESULTS AND DISCUSSION

Protoplast Isolation. The optimum protoplast yield was 2.5×10^6 protoplasts/g fresh weight with a viability of 70% as measured by FDA (Fig. 1, Table 1). Enzyme mixture and concentration were important for optimal protoplast yield and viability. Many types of enzymes were tested, including Cellulysin, Macerase, Rhozyme HP150 (Corning Glass, Corning, NY, USA), Driselase (Kyowa Hakko Kogyo Co., Japan), and Pectolyase Y23 (Kikkoman Shoyu Co., Ltd., Japan). Results were the best with Cellulysin and Macerase combination (Fig. 1).

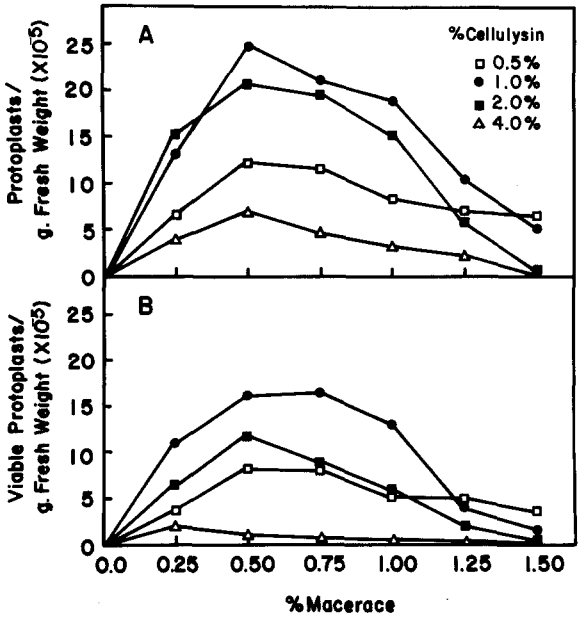

Fig. 1. Effects of enzyme concentration on yield (A) and viability (B) of cotyledon protoplasts isolated from G. hirsutum cv. GSA 78. Cotyledons from 10-day-old seedlings were preplasmolyzed on MSG agar medium for 24 h and then incubated in the enzymes for 16 h.

Protoplast yield increased with the concentration of Cellulysin up to 1% and Macerase up to 0.5%. At higher concentrations, enzyme solutions were toxic and viability of protoplasts was significantly reduced (Fig. 1B). It was important to include nutrients (see MATERIALS AND METHODS) in the enzyme solution to maximize protoplast viability, since the incubation period was relatively long. Calcium chloride was important for stabilizing the protoplast membrane during purification. MES had no effect on protoplast yield, viability, growth, or division (data not shown). Osmotica, including glucose, mannitol, sorbitol, sucrose, and potassium chloride were tested for protoplast preparation. A combination of glucose (2%) and mannitol (7%) was most efficient. An incubation period of 16-18 h at 30°C in darkness produced the largest numbers of protoplasts of high quality (Table 1).

Plants grown in the greenhouse produced inconsistent results for yield and viability of cotyledon and leaf protoplasts. The best donor material was the seeds germinated aseptically on MS medium or in Jiffy-Mix (JPA, Chicago, IL, USA), and incubated at 30°C, in 16 h photoperiod of 4,000 lux. Watts et al. (1974) demonstrated that conditioning of donor plants is critical for successful isolation and culture of leaf protoplasts of Nicotiana tabacum. Similarly, Shepard and Totten (1977) reported that specific plant growth conditions were necessary for sustained growth and development of protoplast cultures of Solanum tuberosum.

Plant genotype and age of the plants were important factors influencing protoplast yield and viability. Optimum protoplast yield and viability was obtained with cotyledons from 10- to 12-day-old seedlings and with leaves from 3-week-old plants. Protoplast release was poor from cotyledons of very young (i.e. younger than 8-10 days) or very old (i.e. older than 2 weeks) seedlings (Table 1). In some genotypes such as Giza 7, seedlings older than 4 weeks produced almost no protoplasts, whereas 10-week-old plants of GSA 78 still yielded reasonable numbers of protoplasts (data not shown). Generally, seedling age was more critical with G. barbadense than G. hirsutum cultivars. Protoplast yield was best for most varieties of both species when the seedlings were growing rapidly. Khasanov and Butenko (1979) reported that 10- to 12-day-old cotyledons were best for cotton protoplasts. David et al. (1982) reported similar results for Pinus pinaster.

Plasmolysis of leaves on MSG medium at 30°C in darkness for 24 h was necessary prior to enzyme treatments for optimum protoplast yield and quality (Table 1). Preplasmolysis of the cotyledons on agar medium rather than liquid medium was important. The nutrient composition of medium did not seem to have much effect, but duration of the treatment had significant effects on yield and viability of protoplasts (Table 1). Vacuum infiltration for 20 min in a vacuum dessicator connected to a water aspirator that allowed gradual infiltration of enzymes was much better than with a water aspirator alone. The latter procedure has been used for protoplast preparation of many systems (Keller et al. 1982) but produced less viable protoplasts of cotton (data not shown).

Generally, cotyledons produced higher numbers of protoplasts with higher viability than did leaves. Cotyledon protoplasts were relatively large (30-35 µm in diameter) (Fig. 2). Most of the freshly isolated protoplasts had their chloroplasts clustered at one pole (Fig. 2a), but after 24 h the chloroplasts were evenly distributed (Fig. 2b). Occasionally some protoplasts probably of vascular origin had a pink or red pigmentation and had no chloroplasts. Leaf protoplasts were smaller in size (20-25 µm) and more uniform.

The procedure developed for cotton cotyledon protoplast preparation was suitable for all lines tested. Therefore, this method could be used for almost all cultivars of cotton.

Table 1. Effects of seedling age, preplasmolysis and incubation periods, and of nutrients and calcium chloride in the enzyme solution on cotyledon protoplast yield and viability.

Cultivar[a]	Variable[b]	No. of protoplasts/g. fresh weight ($x10^{-6}$)	Protoplast viability (%)[c]	No. of viable protoplasts/g. fresh weight ($x10^{-6}$)
GSA 78	Age of seedling (d)			
	4	0.5	49	0.2
	6	1.1	53	0.6
	8	1.5	54	0.8
	10	2.4	72	1.7
	12	2.5	70	1.7
	14	2.2	--	--
	16	1.9	66	1.3
GSA 20	Preplasmolysis period (h)			
	12	1.6	52	0.8
	24	2.1	68	1.4
	48	1.9	59	1.1
	72	0.2	45	0.1
GSA 78	24	2.2	65	1.4
Acala SJ-2	Incubation period (h)			
	4	0.4	75	0.3
	8	1.6	78	1.2
	12	2.0	71	1.4
	16	2.4	73	1.8
	18	2.4	70	1.7
	20	3.1	52	1.6
	24	3.5	45	1.6
GSA 78	18	2.0	65	1.3
Giza 7	18	2.1	70	1.5
SBSI	18	2.5	70	1.8
GSA 78	Nutrients and calcium chloride were present in the enzyme solution[d]	2.4	72	1.7
	Calcium chloride was omitted	1.1	71	0.8
	Nutrients and calcium chloride were omitted	1.0	48	0.5

[a] GSA 20, GSA 78 and Acala SJ-2 are G. hirsutum and Giza 7 and SBSI are G. barbadense cultivars.

[b] In testing a variable, other variables were held constant as follows: age of seedlings 10 d, preplasmolysis period 24 h, incubation period 16 h, and nutrients and calcium chloride were present in the enzyme solution.

[c] Determined by FDA assay (Widholm 1972) immediately after protoplast preparation.

[d] See MATERIALS AND METHODS.

Protoplast Culture. In most of the media tested, some cotyledon protoplasts lost their spherical shape, elongated, and grew in volume after 48 h. However, the best results were obtained with MSm medium. In some lines after 3-4 days in culture 50-80% of the protoplasts regenerated cell walls (Table 2). First divisions were apparent after 4-5 days in culture (Fig. 2c-e) and second and third divisions within two weeks (Figs. 2f and 3). In other lines, the divisions were delayed. Culture density was an important factor influencing protoplast development (Table 2). The optimal culture densities for G. hirsutum cultivars were 4-6 x 10^4 protoplasts/ml and those for G. barbadense cultivars were around 2.5 x 10^4 protoplasts/ml.

In cotyledon protoplasts of G. hirsutum cultivars, budding was very common and cell division rare with the exception of GSA 20 (Fig. 2e-f and Table 2). However, these cells never went beyond the second division and degenerated afterwards. The protoplasts isolated from leaves of G. hirsutum showed budding but regular division did not occur. The differential behavior of cotyledon and leaf protoplasts implies the importance of the donor tissue for protoplast culture, and that various tissues of the same genotype might respond differently in culture.

Protoplasts cultured in the light or at temperatures below 27°C showed budding, but regular division did not occur. It has been proposed that

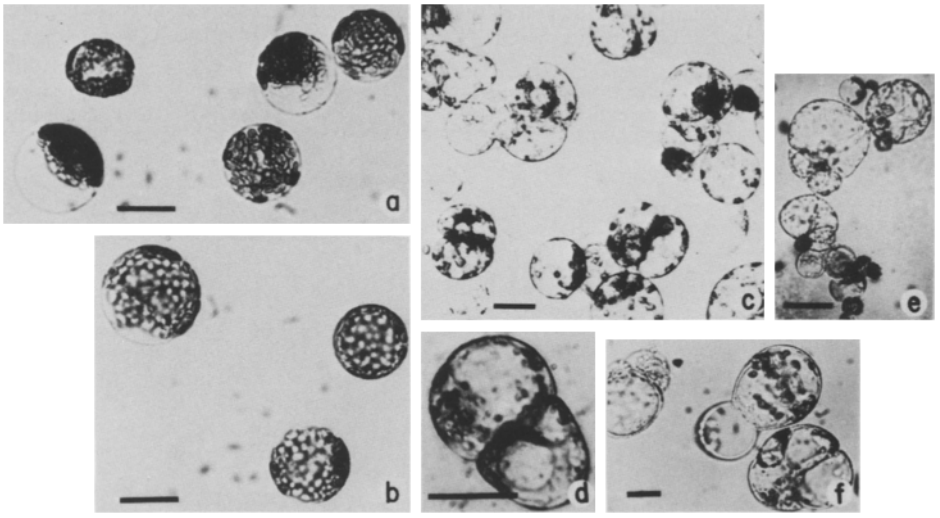

Fig. 2. Development of cotyledon protoplasts of G. hirsutum in culture. Protoplasts were prepared under optimal conditions as explained in MATERIALS AND METHODS. a. Freshly isolated protoplasts showing the chloroplasts at one pole. b. Even distribution of chloroplasts after one day culture of protoplasts. c. Cell wall regeneration and first cell division. d. First division with a complete plate at higher magnification. e. Incomplete cell wall regeneration resulting in separation of cells after budding. f. Budding and second cell division. Bar = 20 µm in all the plates.

Table 2. Effects of culture density on cell wall regeneration, budding, and cell division of cotyledon protoplasts.

Cultivar[a]	Culture[b] density ($x10^4$)	% Cell wall regeneration[c]	% Protoplast budding[d]	% Cell division[d]
Acala SJ-2	2.0	31	45	0
	4.0	45	50	2
	6.0	48	55	2
	8.0	43	51	0
	10.0	45	45	--
	20.0	36	24	1
GSA 20	4.0	55	51	13
GSA 78	2.0	26	53	0
	3.0	50	62	3
	5.0	41	60	2
Giza 7	2.5	75	60	20
SBSI	2.5	80	60	25

[a]Acala SJ-2, GSA 20 and GSA 78 are G. hirsutum and Giza 7 and SBSI are G. barbadense cultivars.

[b]MSm medium was used for culture of protoplasts (see MATERIALS AND METHODS).

[c]Determined by Calcofluor White staining (Galbraith 1981) after 4 d in culture.

[d]Determined after 7 d in culture.

protoplast budding occurs when pectin is not incorporated into the new cell wall (Hanke and Northcote, 1974) and is the result of weakened areas of the cell wall (Fowke and Gamborg, 1980).

Generally, 75-80% of G. barbadense protoplasts regenerated cell walls after 4-5 days in culture, and 20-25% of them entered first division (Table 2) and formed colonies of 5-8 cells within 2 weeks (Fig. 3). El-Shihy and Evans (1983) reported that only 7% of the protoplasts of G. barbadense initiated division and 6% of those embarking upon division formed colonies.

Attempts to obtain microcalli from G. hirsutum protoplasts have not been successful. Different protoplast culture systems such as the agarose bead type culture system of Shillito (1983), the x-plate system of Shepard et al. (1980), the feeder plate

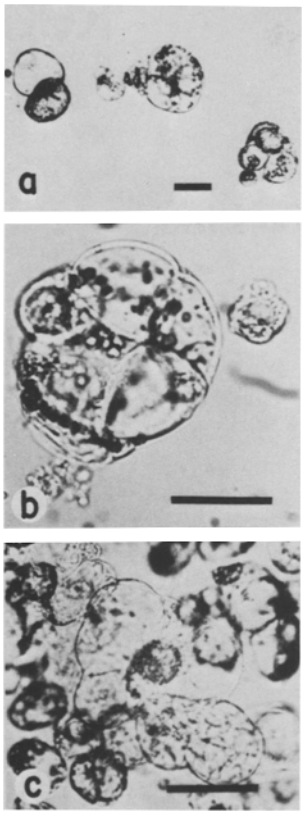

Fig. 3. Cell division and microcolony formation in cotyledon protoplasts of G. barbadense cv. SBSI prepared under optimal conditions. a. Cell division and microcolony formation. b-c. Microcolonies at higher magnification. Bar = 20 µm in all the plates.

system of Horsch and Jones (1980), and a double-layer medium in which liquid medium is placed on agar medium with lower osmoticum and auxin concentrations (unpublished results) did not improve further development of G. hirsutum cells. Growth and division of cotton cells was not sustained by media that have been used for mesophyll protoplast culture of tobacco (Galbraith and Mauch, 1980), cassava (Shahin and Shepard, 1980), lettuce (Engler and Grogan, 1982), haploid tobacco (Caboche, 1980), and potato (Shepard, 1982). Modifications, including the addition of different compounds in the media, e.g. amino acids other than glutamine (5-10 mM each of arginine, lysine, valine, isoleucine, glycine, methionine, threonine, and asparagine), pectin (0.01-0.2%), coconut milk (5-40%), $CaCl_2$ and NH_4NO_3 (10-500 mM of each), and the use of paraffin or mineral oil (Caplin, 1959) to absorb any gossypol produced (Markman and Rzhekhin, 1969) did not help, although the addition of 10 mM putrescine, 0.5 mg/l GA_3, or 1 mg/l pCPA to the MSm medium sometimes kept the protoplasts viable for longer periods.

The combination of cytokinins and auxins used here was the only level of hormones, selected from more than a hundred combinations (i.e., in mg/l BAP, 0.02-2; kinetin, 0.02-2; zeatin, 0.1-2; NAA, 0.3-15; and 2,4-D, 0.2-7), that resulted in cell division of G. hirsutum cotyledon protoplasts. Also, high levels of micro- and macro-elements in the medium were necessary to obtain high percentages of cell division. El-Shihy and Evans (1983) reported that in G. barbadense protoplasts, addition of macro- and micro-elements to MS medium greatly enhanced cell wall regeneration and cell division. These observations suggest some kind of balance between these salts and the hormones needed for division of cotton protoplasts.

ACKNOWLEDGEMENTS

We thank Dr. Bobby Phipps, GroAgri Seed Co., Lubbock, Texas, and Dr. Roberta Smith, Texas A & M University, College Station, Texas, for providing seeds, and Mrs. Julie Nelson for skillful preparation of this manuscript. The senior author is grateful to Dr. David Galbraith, University of Nebraska, Lincoln, NE, where this work was initiated. This is Agrigenetics Advanced Research Division Manuscript No. 45.

REFERENCES

Bhojwani SS, Power JB, Cocking EC (1977) Pl. Sci. Lett. 8: 85-89.
Caboche M (1980) Planta 149: 7-18.
Caplin SM (1959) Amer. J. Bot. 46: 324-329.
David H, David A, Mateille T (1982) Physiol. Plant 56: 108-113.
El-Shihy OM, Evans PK (1983) 6th Int'l Protopl. Symp., Basel, Switzerland, pp. 24-25.
Engler DE, Grogan RG (1982) Pl. Sci. Lett. 28: 223-229.
Finer JJ, Smith RH (1982) Pl. Sci. Lett. 26: 147-151.
Fowke LC, Gamborg OL (1980) Int. Rev. Cytol. 68: 9-51.
Galbraith DW (1981) Physiol. Plant 53: 111-116.
Galbraith DW, Mauch TJ (1980) Z. Pflanzenphysiol. 98: 129-140.
Hanke DE, Northcote DH (1974) J. Cell Sci. 14: 29-50.
Horsch R, Jones G (1980) In Vitro 16: 103-108.
Keller WA, Setterfield G, Douglas G, Gleddie S, Nakamura C (1982) In: Tomes DT, Ellis BE, Harney PM, Kasha KJ, Peterson RL (eds), Application of plant cell and tissue culture to agriculture & industry, The University of Guelph, Guelph, Canada, pp 81-115.
Khasanov MM, Butenko RG (1979) Fiziologiya Rastenit 26: 77.
Markman AL, Rzhekhin VP (1969) Gossypol and its derivatives. Israel Prog. Scient. Transl. LTD., Jerusalem.
Murashige T, Skoog F (1962) Physiol. Plant 15: 473-497.
Racusen RH, Kinnersley AM, Galston AW (1977) Science 198: 405-406.
Shahin E, Shepard JF (1980) Pl. Sci. Lett. 17: 459-465.
Shepard JF (1982) Pl. Sci. Lett. 26: 127-132.
Shepard JF, Bidney D, Shahin E (1980) Science 208: 17-24.
Shepard JF, Totten RE (1977) Plant Physiol. 60: 313-316.
Shillito RD, Paszkowski J, Potrykus I (1983) Plant Cell Reports 2: 244-247.
Uchimiya H, Murashige T (1974) Pl. Physiol. 54: 936-944.
Watts JM, Motoyoshi F, King JM (1974) Ann. Bot. 38: 667-671.
Widholm JM (1972) Stain Technol. 47: 189-194.